

WHAT IS CLAIMED IS:

1. A fibronectin type III (Fn3) molecule, wherein the Fn3 comprises a stabilizing mutation as compared to a wild-type Fn3.
2. The Fn3 of claim 1, wherein the stabilizing mutation comprises at least one aspartic acid (Asp) residue that has been deleted or substituted with at least one other amino acid residue.
3. The Fn3 of claim 2, wherein Asp 7 or Asp 23, or both, have been deleted or substituted with at least one other amino acid residue.
4. The Fn3 of claim 3, wherein Asp 7 or Asp 23, or both, have been substituted with an asparagine (Asn) or lysine (Lys) residue.
5. The Fn3 of claim 1, wherein the stabilizing mutation comprises at least one glutamic acid (Glu) residue that has been deleted or substituted with at least one other amino acid residue.
6. The Fn3 of claim 5, wherein Glu 9 has been deleted or substituted with at least one other amino acid residue.
7. The Fn3 of claim 6, wherein Glu 9 has been substituted with an asparagine (Asn) or lysine (Lys) residue.
8. The Fn3 of claim 2, wherein Asp 7, Asp 23, and Glu 9 have been deleted or substituted with at least one other amino acid residue.
9. A fibronectin type III (Fn3) polypeptide monobody comprising a plurality of Fn3 β -strand domain sequences that are linked to a plurality of loop region sequences,
wherein one or more of the monobody loop region sequences vary

09000412 071104

wherein the β -strand domains of the monobody have at least a

wherein the Fn3 comprises a stabilizing mutation.

2. *Leaves*

16. The monobody of claim 9, wherein the monobody loop region sequences vary from the wild-type Fn3 loop region sequences by the deletion or replacement of at least 2 amino acids.
17. The monobody of claim 9, wherein the monobody loop region sequences vary from the wild-type Fn3 loop region sequences by the insertion of from 3 to 25 amino acids.
18. An isolated nucleic acid molecule encoding the polypeptide monobody of claim 1.
19. An expression vector comprising an expression cassette operably linked to the nucleic acid molecule of claim 18.
20. The expression vector of claim 19, wherein the expression vector is an M13 phage-based plasmid.
21. A host cell comprising the vector of claim 19.
22. A method of preparing a fibronectin type III (Fn3) polypeptide monobody comprising the steps of:
- providing a DNA sequence encoding a plurality of Fn3 β -strand domain sequences that are linked to a plurality of loop region sequences, wherein at least one loop region contains a unique restriction enzyme site, and wherein at least one of the plurality of Fn3 β -strand domain sequences are more stable at neutral pH than wild-type Fn3;
 - cleaving the DNA sequence at the unique restriction site;
 - inserting into the restriction site a DNA segment known to encode a peptide capable of binding to a specific binding partner (SBP) or a transition state analog compound (TSAC) so as to yield a DNA molecule comprising the insertion and the DNA sequence of (a);

Sub A3
Cont

09603442 07.1.10.1

dict
of
the
loop
region

and

- d) expressing the DNA molecule so as to yield polypeptide monobody.

23. A method of preparing a fibronectin type III (Fn3) polypeptide monobody comprising the steps of:

- (a) providing a replicatable DNA sequence encoding a plurality of Fn3 β -strand domain sequences that are linked to a plurality of loop region sequences, wherein the nucleotide sequence of at least one loop region is known, and wherein at least one of the plurality of Fn3 β -strand domain sequences are more stable at neutral pH than wild-type Fn3;
- (b) preparing polymerase chain reaction (PCR) primers sufficiently complementary to the known loop sequence so as to be hybridizable under PCR conditions, wherein at least one of the primers contains a modified nucleic acid sequence to be inserted into the DNA;
- (c) performing polymerase chain reaction using the DNA sequence of (a) and the primers of (b);
- (d) annealing and extending the reaction products of (c) so as to yield a DNA product; and
- (e) expressing the polypeptide monobody encoded by the DNA product of (d).

24. A method of preparing a fibronectin type III (Fn3) polypeptide monobody comprising the steps of:

- a) providing a replicatable DNA sequence encoding a plurality of Fn3 β -strand domain sequences that are linked to a plurality of loop region sequences, wherein the nucleotide sequence of at least one loop region is known, and wherein at least one of the plurality of Fn3 β -strand domain sequences are more stable at neutral pH than wild-type Fn3;

Sub 3
a3
cont.

FOI b7E b7C b7D

11

- 00004423
"071301"
L
a3
Cont

95

acid loop from residue 70 to residue 75 of the wild-type Fn3 loop region, comprising from 3 to 25 amino acids.

acid library of claim 26, comprising from 6 to 75 amino acids, located in the BC loop region of the sp.

acid library of claim 26, comprising from 6 to 75 amino acids, located in the DE loop region of the sp.

acid library of claim 26, comprising from 6 to 75 amino acids, located in the FG loop region of the sp.

acid library of claim 26, comprising from 6 to 75 amino acids, located in the AB loop region of the sp.

acid library of claim 26, comprising from 6 to 75 amino acids, located in the CD loop region of the sp.

28. The variegated nucleic acid library of claim 26, wherein the loop region sequences vary from the wild-type Fn3 loop region sequences by the deletion or replacement of at least 2 amino acids.
29. The variegated nucleic acid library of claim 26, wherein the monobody loop region sequences vary from the wild-type Fn3 loop region sequences by the insertion of from 3 to 25 amino acids.
30. The variegated nucleic acid library of claim 26, wherein a variegated nucleic acid sequence comprising from 6 to 75 nucleic acid bases is inserted in any one of the loop regions of the species.
31. The variegated nucleic acid library of claim 26, wherein the variegated sequence is constructed so as to avoid one or more codons selected from the group consisting of those codons encoding cysteine or the stop codon.
32. The variegated nucleic acid library of claim 26, wherein the variegated nucleic acid sequence is located in the BC loop.
33. The variegated nucleic acid library of claim 26, wherein the variegated nucleic acid sequence is located in the DE loop.
34. The variegated nucleic acid library of claim 26, wherein the variegated nucleic acid sequence is located in the FG loop.
35. The variegated nucleic acid library of claim 26, wherein the variegated nucleic acid sequence is located in the AB loop.
36. The variegated nucleic acid library of claim 26, wherein the variegated nucleic acid sequence is located in the CD loop.

[illegible]

37. The variegated nucleic acid library of claim 26, wherein the variegated nucleic acid sequence is located in the EF loop.
38. A peptide display library derived from the variegated nucleic acid library of claim 26.
39. A peptide display library of claim 38, wherein the peptide is displayed on the surface of a bacteriophage or virus. *specis*
40. A peptide display library of claim 39, wherein the bacteriophage is M13 or fd.
41. A method of identifying the amino acid sequence of a polypeptide molecule capable of binding to a specific binding partner (SBP) so as to form a polypeptide:SSP complex wherein the dissociation constant of the the polypeptide:SBP complex is less than 10^{-6} moles/liter, comprising the steps of:
- providing a peptide display library according to claim 39;
 - contacting the peptide display library of (a) with an immobilized or separable SBP;
 - separating the peptide:SBP complexes from the free peptides,
 - causing the replication of the separated peptides of (c) so as to result in a new peptide display library distinguished from that in (a) by having a lowered diversity and by being enriched in displayed peptides capable of binding the SBP;
 - optionally repeating steps (b), (c), and (d) with the new library of (d); and
 - determining the nucleic acid sequence of the region encoding the displayed peptide of a species from (d) and deducing the peptide sequence capable of binding to the SBP.
42. A method of preparing a variegated nucleic acid library encoding Fn3

Sub 3
a3
Cont

FOI b7E b7C b7D b7F b7G b7H b7I b7J b7K b7L b7M b7N b7O b7P b7Q b7R b7S b7T b7U b7V b7W b7X b7Y b7Z

XI

XII

polypeptide monobodies having a plurality of nucleic acid species each comprising a plurality of loop regions, wherein the species encode a plurality of Fn3 β -strand domain sequences that are linked to a plurality of loop region sequences, wherein one or more of the loop region sequences vary by deletion, insertion or replacement of at least two amino acids from corresponding loop region sequences in wild-type Fn3, and wherein the β -strand domain sequences of the monobody have at least a 50% total amino acid sequence homology to the corresponding amino acid sequences of β -strand domain sequences of the wild-type Fn3, and wherein the Fn3 comprises a stabilizing mutation β -strand domain, comprising the steps of

- a) preparing an Fn3 polypeptide monobody having a predetermined sequence;
- b) contacting the polypeptide with a specific binding partner (SBP) so as to form a polypeptide:SBP complex wherein the dissociation constant of the the polypeptide:SBP complex is less than 10^{-6} moles/liter;
- c) determining the binding structure of the polypeptide:SBP complex by nuclear magnetic resonance spectroscopy or X-ray crystallography; and
- d) preparing the variegated nucleic acid library, wherein the variegation is performed at positions in the nucleic acid sequence which, from the information provided in (c), result in one or more polypeptides with improved binding to the SBP.

43. A method of identifying the amino acid sequence of a polypeptide molecule capable of catalyzing a chemical reaction with a catalyzed rate constant, k_{cat} , and an uncatalyzed rate constant, k_{uncat} , such that the ratio of k_{cat}/k_{uncat} is greater than 10, comprising the steps of:

- a) providing a peptide display library according to claim 39;
- b) contacting the peptide display library of (a) with an immobilized or separable transition state analog compound (TSAC) representing the approximate molecular transition state of the chemical

05904412-071101
 Sub 3
 Cont

111

reaction;

- c) separating the peptide:TSAC complexes from the free peptides;
- d) causing the replication of the separated peptides of (c) so as to result in a new peptide display library distinguished from that in (a) by having a lowered diversity and by being enriched in displayed peptides capable of binding the TSAC;
- e) optionally repeating steps (b), (c), and (d) with the new library of (d); and
- f) determining the nucleic acid sequence of the region encoding the displayed peptide of a species from (d) and hence deducing the peptide sequence.

44. A method of preparing a variegated nucleic acid library encoding Fn3 polypeptide monobodies having a plurality of nucleic acid species each comprising a plurality of loop regions, wherein the species encode a plurality of Fn3 β -strand domain sequences that are linked to a plurality of loop region sequences, wherein one or more of the loop region sequences vary by deletion, insertion or replacement of at least two amino acids from corresponding loop region sequences in wild-type Fn3, and wherein the β -strand domain sequences of the monobody have at least a 50% total amino acid sequence homology to the corresponding amino acid sequences of β -strand domain sequences of the wild-type Fn3, and wherein the Fn3 comprises a stabilizing mutation β -strand domain, comprising the steps of
- a) preparing an Fn3 polypeptide monobody having a predetermined sequence, wherein the polypeptide is capable of catalyzing a chemical reaction with a catalyzed rate constant, k_{cat} , and an uncatalyzed rate constant, k_{uncat} , such that the ratio of k_{cat}/k_{uncat} is greater than 10;
 - b) contacting the polypeptide with an immobilized or separable transition state analog compound (TSAC) representing the approximate molecular transition state of the chemical reaction;
 - c) determining the binding structure of the polypeptide:TSAC

Sub A3
Cont

FOI b7D b7C b7E b7F b7G b7H b7I b7J b7K b7L b7M b7N b7O b7P b7Q b7R b7S b7T b7U b7V b7W b7X b7Y b7Z

7/1/1

99

nuclear magnetic resonance
hy; and
variegated nucleic acid lib
s performed at positions in
the information provided in
with improved binding to
side identified by the metho
side identified by the metho
the amino acid sequence of
a specific binding partner
complex wherein the dissociat
complex is less than 10^{-6} mole
y of claim 39.

the amino acid sequence of
a chemical reaction with a
ed rate constant, k_{uncat} , such
mprising the peptide displa
d by using the kit of claim
d by using the kit of claim

45. An isolated polypeptide identified by the method of claim 41.
46. An isolated polypeptide identified by the method of claim 43.
47. A kit for identifying the amino acid sequence of a polypeptide molecule capable of binding to a specific binding partner (SBP) so as to form a polypeptide:SSP complex wherein the dissociation constant of the the polypeptide:SBP complex is less than 10^{-6} moles/liter, comprising the peptide display library of claim 39.
48. A kit for identifying the amino acid sequence of a polypeptide molecule capable of catalyzing a chemical reaction with a catalyzed rate constant, k_{cat} , and an uncatalyzed rate constant, k_{uncat} , such that the ratio of k_{cat}/k_{uncat} is greater than 10, comprising the peptide display library of claim 39.
49. A polypeptide derived by using the kit of claim 47.
50. A polypeptide derived by using the kit of claim 48.

701